Alteration of AP-endonuclease1 expression in curcumin-treated fibrotic rats

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ABSTRACT

Background. Apurinic/apyrimidinic endonuclease1/ redox factor-1 (APE1/Ref-1) is a multifunctional protein involved in DNA base excision repair and redox regulation of many transcription factors. It is an important pro-survival protein activated in response to oxidative stress. Increased level of this essential redox sensitive protein correlates closely with cellular survival against oxidative insults. Curcumin (diferuloylmethane) a naturally occurring compound derived from turmeric has attracted interest because of its anti-inflammatory, anti-oxidative, and chemopreventive activities. **Material and methods.** The current study evaluates the *in vivo* role of curcumin in protecting and treating liver injury and fibrogenesis caused by carbon tetrachloride (CCl_4) in rats. It also addresses the possible involvement of the multifunctional protein APE1 in hepatoprotection. Analysis of APE1 expression was performed at mRNA and protein levels by reverse transcriptase (RT)-PCR and western blotting respectively. Profile of HSCs-activation related genes were assayed by RT-PCR and pro-inflammatory cytokines levels were determined by enzyme-linked immune assays. **Results.** Here we show that oral administration of curcumin was accompanied by a robust increase in APE1 protein and mRNA levels, and improved the histological architecture of rat liver. In addition, curcumin attenuated oxidative stress by increasing the content of hepatic glutathione within normal values, leading to the reduction in the level of lipid hydroperoxide. Curcumin remarkably suppressed inflammation by reducing levels of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), nuclear factor-kappa B (NF-κB) and interleukin-6 (IL-6). It also inhibited hepatic stellate cells (HSCs) activation by elevating the level of PPARγ and reducing the abundance of transforming growth factor-β (TGF-β). We found that oral administration of curcumin at 200 mg/kg dose not only protected against CCl_4-induced hepatic injury, but also resulted in more than two-fold induction of APE1 protein expression in CCl_4-induced rat group. **Conclusions.** It can be concluded that curcumin reduced markers of liver damage in rats treated with CCl_4, with concomitant elevation in APE1 protein level indicating a possible protective effect with unknown mechanism. The induction of DNA repair enzymes may be an important and novel strategy for hepatic protection against oxidative injury.

Key words. Apurinic/apyrimidinic endonuclease 1. Hepatic stellate cells. Curcumin. Tumor necrosis factor-α (TNF-α). Nuclear factor-kappaB (NF-κB). Transforming growth factor-β (TGF-β).

INTRODUCTION

The pathogenesis of liver fibrosis is not yet completely understood. Hepatic fibrosis is due to an imbalance in extracellular matrix (ECM) breakdown and deposition.[1-3] Research over the past two decades has established that hepatic stellate cells (HSCs) are the primary ECM-producing cell type during hepatic fibrogenesis.[1,2] HSCs activation, characterized by enhanced cell proliferation, overproduction of ECM and *de novo* synthesis of α-smooth muscle actin, is triggered by the release of mitogenic platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) from activated HSCs and fibrogenic transforming growth factor (TGF-β1), mostly from Kupffer cells[1,2] as well as the dramatic down-expression of peroxisome proliferator-activated receptor-γ (PPARγ).[4-6] The alteration in PPARγ activity plays an important role in HSCs activation.[7,8]

Oxidative stress has been recently recognized as a fundamental factor in the pathogenic changes observed in liver fibrosis. It plays an important role in pathophysiological changes that progress to liver cirrhosis.

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Cellular response to oxidative stress is a highly regulated and complex biological process.[9] APE1 is a ubiquitous and remarkably multifunctional protein. It plays a central role in the base excision repair (BER) pathway for damaged bases and DNA single-strand breaks induced by reactive oxygen species (ROS) and alkylating agents, and hence is an attractive target for possible hepatoprotection, as it is a critical component of the oxidative stress up-regulated proteins. To prevent ROS accumulation, cells have several antioxidant systems including enzymes and redox-sensitive molecules (Trx, APE1/Ref-1) that protect the cells from oxidative stress.[10] Activation of APE1/Ref-1 increases the binding of oxidative stress regulating transcription factors (Hif-1, p53, Ap-1). In aged rats, APE1/Ref-1 is decreased and responses to stress in the brain are defective.[11,12] There are many studies on the relationship between p53 and APE1/Ref-1.[13,14]

Discoveries during the past 2 decades have begun to clarify genetic regulation of fibrogenesis and have thereby indicated areas of potential therapeutic intervention. Therefore, the development of interventions designed to impede or reverse hepatic fibrosis is of high priority and is urgently needed. Most evolving antifibrogenic therapies will be aimed at inhibiting HSCs activation. The antioxidant curcumin, a polyphenolic antioxidant purified from turmeric, is an important and widely used spice of the diet in several countries. It has received attention as a promising dietary supplement for the treatment of hepatic fibrogenesis.[15-19]

In vitro, curcumin induces apoptosis and inhibits activation and proliferation of HSCs. In addition, it prevents formation and development of the extracellular matrix by inhibiting collagen α1(I), fibronectin and a-smooth muscle actin gene expression, by enhancing matrix metalloproteinase-2 and -9 expression and suppressing CTGF expression.[19-21] In addition to several intracellular signaling pathways are modulated by curcumin in HSCs.[19,20]

The current study was designed to investigate the prophylactic effects and the mechanisms of curcumin on liver fibrosis in rats, and the concomitant modular expression of APE1 and thus to explore the possible relationship between them.

MATERIALS AND METHODS

Animals

Seventy male Sprague-Dawley rats, weighing 90-120 g were used in this study. The animals were maintained under controlled temp (25 ± 2 °C) and constant periodic conditions (12 h light, 12 h dark). The dams had free access to water and to standard food (20% protein, 54% carbohydrates, 4% lipid, 7% ash, 10% moisture). The principles of laboratory animal's care were followed in all experimental protocols and were approved by ethics committees for animal's research at Faculty of Medicine, Alexandria University.

Induction of hepatic injury and fibrogenesis using CCl₄

Rats were divided into three groups: control group (group 1) received subcutaneous injection of empty vehicle), CCl_4-induced group (group 2) received subcutaneous injection of CCl_4 (0.3 mL/kg body weight) once per week for 4 weeks followed by twice injections weekly for 12 weeks) and pre-cur & CCl_4-induced (group 3) where rats were administered curcumin (200 mg/kg) by gavages tube twice weekly for 4 weeks before CCl_4 administration as in group 2.

At the end of fibrosis-induction period, 10 rats from groups 2 and 3 were treated by curcumin administration (200 mg/kg; suspended in sterile PBS) for 8 weeks.

The study aimed to investigate the molecular changes caused by CCl_4-induced fibrosis and curcumin treatment in a dose- and time-dependent manner; therefore three rats from each group (groups 1, 2 and 3) were sacrificed by cervical dislocation at time intervals of 6, 10 and16 weeks following CCl_4 injection and at weeks 20 and 24 following curcumin treatment (i.e. after 4 and 8 weeks of curcumin treatment respectively). Blood sample from each sacrificed rat was collected by heart puncture. Sera were collected and stored at -80 °C for liver function biochemical analyses (ALT, AST, albumin, total protein, and bilirubin).

Liver tissues were removed from each sacrificed rat; small portion was fixed in 10% neutral-buffered formalin (NBF) for histological analysis and the remaining tissue was immediately frozen in liquid nitrogen then stored at -80 °C.

Histopathological analysis

The fixed liver tissues in formalin were dehydrated in ascending grades of alcohol, and then cleaned by immersing the tissues in xylene for 1 h (three times), followed by impregnation in melted paraffin, in wax, then dried at oven for 1 h. Then the specimens were embedded in paraffin and were left to so-

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lidify at room temperature. Using a rotatory microtome, multiple sections of 5 μm thick were cut and were mounted on clean glass slides. All sections were stained with hematoxylin and eosin (H&E) and examined for any histopathological changes.[22] Pathological diagnosis of each liver specimen was assessed and graded from 0 to VI by two pathologists in a blinded manner according to the criteria described by Wang, *et al.*[23]

Analyses of hepatocytic death and hepatic injury parameters

Blood was collected from each rat by heart puncture when sacrificed. After coagulation, sera were collected and stored at -20 ºC for further analyses. Activities of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein and albumin levels in sera were analyzed using routine laboratory assay kits.

Determination of lipid peroxidation

Part liver tissue (10% w/v) was washed with saline solution, minced and homogenized in ice cooled buffer 50 mm potassium phosphate pH 7.5, the homogenate was centrifuged at 10,000 rpm for 20 min at 4 ºC , and the supernatant was used for the assay. Level of lipid per oxidation was determined based on Ohkawa, *et al.*,[24] colorimetric method. Thiobarbiuitic acid (TAB) reacts with malondialdehyde (MDA) in acidic medium at temp 95 ºC for 30 min to form thiobarbiuitic acid reactive product the absorbance of the resultant pink product can be measured at 534 nm and is expressed in nmol/g.tissue.

Determination of reduced glutathione (GSH) and glutathione peroxidase activity (GPX)

Part liver tissue (10% w/v) was washed with saline solution, minced and homogenized in ice cooled buffer1.15% kcl, 0.01 M sodium phosphate buffer pH 7.4 the homogenate was centrifuged at 10,000 rpm for 20 min at 4 ºC and the supernatant was used for determination of GSH , GPX and protein content. The concentration of GSH was determined as described by Eliman;[25] Eliman's reagent [5,5', dithiobis (2-nitrobenzoic acid), DTNB] was reacted with GSH giving 2-nitro-5-thiobenzoic acid , a yellow colored product with a maximum absorbance at 412 nm, GSH level is expressed as mg/g liver.

Total glutathione peroxidase (Gpx) was determined spectrophotometrically using cumene hydroperoxide as substrate. The excess amount of reduced glutathione was determined using Ellman method. Gpx activity were calculated as (μg/min/mL), the consumed GSH (μg/mL)/time of incubation (min).

Total RNA isolation and reverse transcriptase polymerase reaction (RT-PCR)

Total RNA was extracted from frozen liver tissues according to Chomczynski and Sacchi procedure[26] using GStractTM RNA Isolation Kit II Guanidinium Thiocyanate method. The extracted dried pellets were resuspended in 50-100 μl RNAase free H_2O and stored at -80 ºC. Alterations in the steady–state mRNA levels of genes relevant to hepatic stellate cells (HSCs) activation and fibrosis development in CCl_4 induced rats is determined using reverse-transcriptase PCR analysis. Using one-step RT-PCR (RT/PCR Master Mix. Gold Beads, BIORON) reaction, the cDNA was synthesized and used for amplification of target gene(s) using specific primer sets. Briefly, total RNA (1-3 μg) and random primer (3 μM) mixture were denatured at 70 ºC for 5 min and placed on ice. The incubated mixture was added to the RT/PCR Gold mix that contains all the components necessary for cDNA synthesis and amplification in one tube. The cDNA synthesis reaction was performed at 42 ºC for 60 min then 5 min at 94 ºC for RTase inactivation. The primers then subjected to PCR cycles. Annealing temperature and time need to be optimized for each primer/template combination. We investigated the expression of pro-fibrotic genes that is induced by activated hepatic stellate cells (HSCs) (TGF-β, CTGF, TIMP-1, STAP and α-SMA) *vs.* anti-fibrotic genes (MMP-2 and PPARγ) as well as the expression of rat albumin, APE1 and p53.

The sequences of the primers set for

- **TTMP:** F-GCCCCAACCCACCCACAGA- R-TTTGCAAGGGATGGCTGAACAG (405 bp).
- **MMP-2:** F-GAGAAAAGCGCAGCGGAGTGACG- R- TTCCCCCGCAAGCCCAAGTG (145 bp).
- **CTGF:** F-ATCCCTGCGACCCACACAAG- R-CAACTGCTTTGGAAGGACTCGC (450 bp).
- **TGFβ-1:** F-CGGGAAGCAGTGCCAGAA- R-TGCTCCACAGTTGACTTGAATCTC (470 bp).
- **APE-1:** F-CTGCCTGGACTCTCTCATCAATAC- R-CCTCATCGCCTATGCCGTAAG (200 bp).
- **p53:** F-CACAGTCGGATATGAGCATC- R-GTCGTCCAGATACTCAGCAT (600 bp).

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- α-**SMA:** F-ACTGGGACGACATGGAAA AG- R-CATCTCCAGAGTCCAGCACA (280 bp).
- **STAP:** F-ATGGAGAAAGTGCCGGGCGAC- R-TGGCCCTGAAGAGGGCAGTGT (340 bp).
- **Rat-albumin:** F-GCTCAGAGACTGCCCTGTGT- R-ACAAGGTTTGGCCCCTCAGT (400 bp).
- **GAPDH:** F-TGCTGGTGCTGAGTATGTCG- R-ATTGAGAGCAATGCCAGCC (646 bp).

Nuclear proteins extraction

Nuclear extract was prepared as described by Schreiber *et al.*[27] 500 mg of frozen tissue was homogenized in 5 mL of ice-cold extraction buffer A (0.6% NP-40, 150 mM NaCl, 10 mM HEPES pH 6.9, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 2,000 rpm for 30 s at 0 ºC. After incubation for 5 min on ice, the supernatant was centrifuged at 5,000 rpm for 5 min at 0 ºC. The supernatant is the cytoplasmic fraction was collected and stored at -80 ºC. The precipitate was resuspended in extraction buffer B (25% glycerol, 420 mM NaCl, and 20 mM HEPES pH7.9, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5mM phenylmethylsulfonyl fluoride, 5 mg/mL each of aprotinin, leupeptin, and pepstatin). After being kept on ice for 20 min, it was transferred to a centrifuge tube and centrifuged at 12,000 rpm for 25 min at 0 ºC. The supernatant was transferred to a new tube and stored at -80 ºC.

Cytokines determination

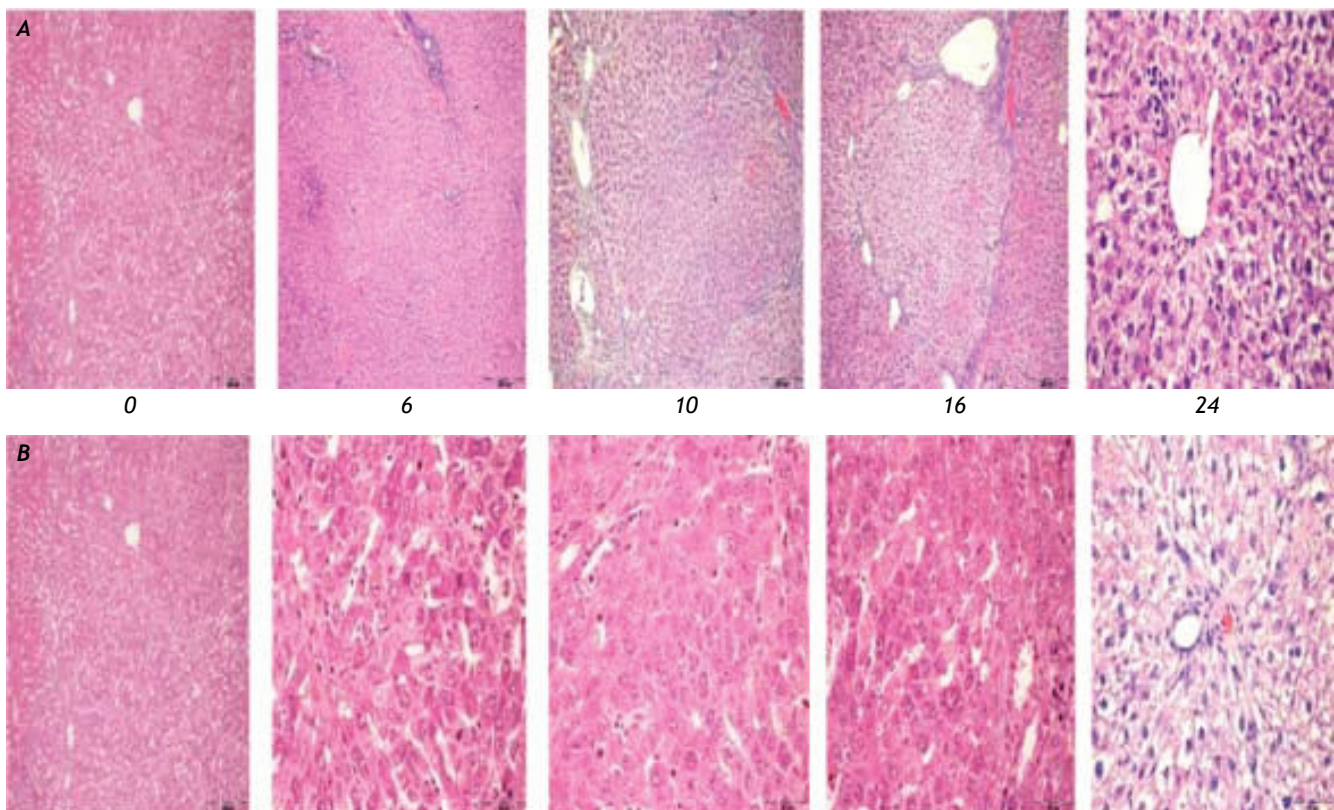IL-6, TNF-α, and TGF-β levels were assayed in total cell extracts prepared form liver tissues and

Figure 1. *Representative microscopic photograph of liver of CCl₄-induced or curcumin-treated rats by hematoxylin-eosin (H&E) staining. **A-0 to A-16** shows the histological findings of the experimental rats of group 2 that was fibrosis-induced with CCl₄ injection for 16 weeks. The results are represented at time intervals of 0, 6, 10, and 16 of CCl₄ injection and at week 24 which represents the result after 8 weeks of curcumin treatment of fibrotic liver. (0) Represents rat' liver section of normal control, **A-24** show fibrosis-induced liver that has been treated with curcumin for 8 weeks. **B-0 toB-16** shows results of group 3 that was pre-cur & CCl₄-induced for 16 weeks. Treatment with curcumin before CCl₄ injection has protective effect on rat liver as represented in figure **B** during the follow up weeks. **B-24:** Sections of curcumin pre-treated, fibrosis-induced then curcumin treated rats for 8 weeks (week 24) showing thick wall of portal track collapsed with congested hepatocytes cell forming pseudo nodules, rest of hepatocytes was vacuolated.*

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sera samples. Cytokines levels were determined using commercially available kits. The analyses were performed according to the manufactures' guidelines. IL-6 level assayed using BioLegend ELISA MAX™ Set Standard Rat IL-6 Kit (Cat.430502). TNF-α was determined using Rat TNF-α Prprotech kit (cat# 900-K25) and TGF-β using Quantikine kit (Cat# MB100B). Nuclear factor-κB p50/65 DNA-binding activity in nuclear extracts of hepatic tissue samples was evaluated. Analysis was performed according to manufacturer's protocol guidelines using commercial Kit (NF-κB p50/65 Transcription Factor Assay Colorimetric. Cat. No. SGT510 Themicon International Inc. Temecula, CA, USA).

Western blotting

According to the method described by Burnette,[28] APE1 and p53 immunoblots were performed on prepared cytosolic and total cell extracts respectively. Briefly, 50 μg protein lysates from each sample were separated on 12% SDS-PAGE then transferred to a nitrocellouse membrane. Primary antibody to APE1 (rabbit Polyclonal anti-APE1) and p53 (sc-DO-1, Santa Cruz Biotechnology) was used. Then incubated with HRP-conjugated secondary antibodies (Santa Cruz; diluted).

Hydroxyproline (hyp) assay in rat liver

A colorimetric assay was performed as described by Nakamura, et al.[29] Briefly, lyopholized liver sections (0.5 g) were hydrolyzed for 20 h in 6 mol/L HCl at 100 °C, redissolved in water, and centrifuged to remove any impurities. Samples were incubated for 10 min in 0.05 mol/L chloramine-T (Fisher, Fair Lawn, NJ, USA) at room temperature, followed by 15-min incubation in Ehrlich's-perchloric acid solution at 65 °C. Sample absorbencies were assessed at 560 nm and resulting values compared to a hyp standard curve. Each sample was assayed in duplicate. The hyp content was expressed as micrograms per gram of wet liver.

Statistical analysis

All experiment was repeated two or more times independently and typically graph are represented. In some cases data are presented as mean ± SD. Data were analyzed by student T-test and different considered significant at $p < 0.05$ or at $p < 0.01$.

RESULTS

Pre-treatment with curcumin ameliorated CCl$_4$-induced liver injury through apoptosis

Representative microscopic photographs of liver sections stained with Hematoxylin-eosin (H&E) staining of CCl$_4$-induced and pre-cur & CCl$_4$-induced groups then treated are shown in figure 1. We assessed the fibrosis degree (score) according to the pathological index described by Wang, et al.[23] Stained and examined liver sections of CCl$_4$-induced rats were graded from score 0 (at week zero, control), II (at week 6), III (at week 10) and from IV to V, (at week 16). At score IV-V the CCl$_4$-injection was stopped and curcumin treatment was started.

Pre-treatment with curcumin for 4 weeks has protective effect on rat liver as represented in the stained sections in figure 1-B where the fibrosis grade assessment was I at week 6, II at week 10, III to IV at week 16 of CCl$_4$-injection compared to advanced progression that has been observed in CCl$_4$-induced rats of group 2. Appearance of area of apoptotic cells in different stages of pre-cur & CCl$_4$-induced induced group was observed as well. Sections of fibrotic rats (group 2) that has been treated with curcumin for 8 weeks indicated portal track collapsed with congested hepatocytes cell forming pseudo nodules, rest of hepatocytes was vacuolated.

The general conditions of rats improved after curcumin treatment within 14 days as assessed by change in the body weight and hyp content. During fibrogenesis induction, the body weight of group 2 rats dramatically reduced within the first 6 weeks (the mean loss body mass was 35.40 ± 8.34 g) and then slowly increased within 6-16 week (increased by 19.44 ± 6.78 g). From week 20, the body weight of rats slightly increased in treatment group with no obvious changes in control group, showing that rats become adapted to toxicity. In addition, hyp content significantly ($p < 0.05$) attenuated in wet liver of pre-cur & CCl$_4$-induced group to 396.8 ± 116.3 mg/g liver compared to 566.0 ± 237.4 mg/g liver in CCl$_4$-induced.

Curcumin reduced CCl$_4$-induced hepatocytes toxicity and injury

Curcumin pre-treatment ameliorated the significant elevation in sera ALT and AST activities compared to CCl$_4$ injected rats without curcumin

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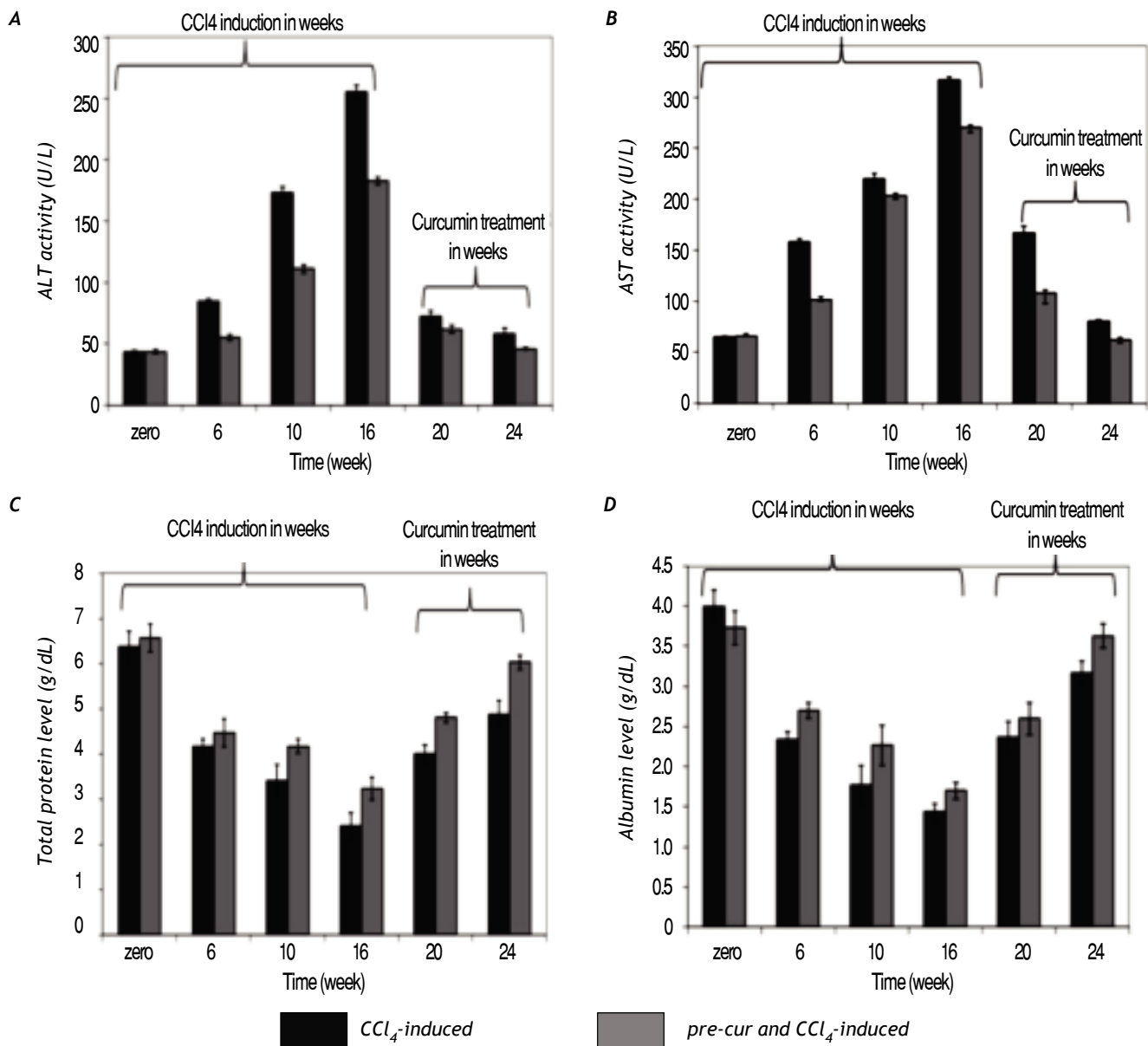
Figure 2. *Changes in liver function indices. The activities of serum AST and ALT as well as the total protein and albumin levels were analyzed during CCl$_4$-induced fibrosis and curcumin. A significant elevation in AST and ALT activities were observed in all groups. In pre-cur & CCl$_4$-induced rat curcumin protected rat liver from CCl$_4$-induced in serum elevated ALT and AST activities as well as reduction in total protein and albumin levels compared to the CCl$_4$-induced group without curcumin pre-treatment (group 2; CCl$_4$-induced). Curcumin administration for 4 and 8 weeks (at weeks 20 and 24), restored hepatocytes function as indicated by significant elevation (p < 0.05) in sera total proteins and albumin levels as well.*

pre-treatment (Figure 2A and 2B). At weeks 20 and 24 (curcumin treatment for 4 and 8 weeks) the results indicated significant (p< 0.05) reduction in the ALT and AST serum activities in both groups. Also pre-treatment with curcumin induces less reduction in total serum protein and albumin levels compared to CCl$_4$-induced rats (Figure 2C and 2D). Alterations in albumin mRNA level as a marker of

hepatocytes synthetic function was found to be highly reduced in liver tissues of CCl$_4$-induced rats compared to pre-cur & CCl$_4$-induced rats (Figure 7). Curcumin treatment for 4 and 8 weeks of fibrotic liver, restored hepatocytes function as indicated by significant elevation (p< 0.05) in serum tissue expression of at albumin mRNA level in groups 2 and 3.
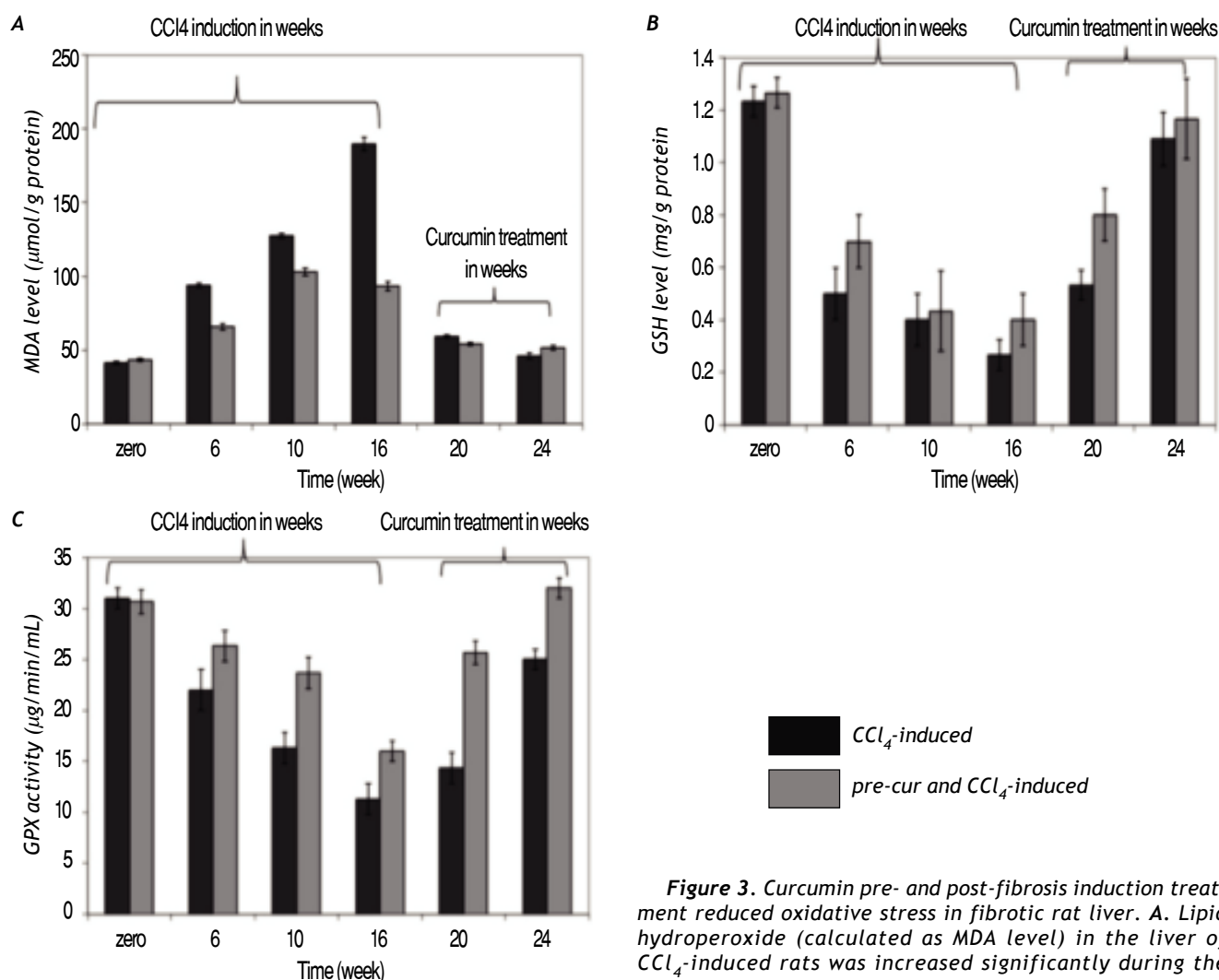
Figure 3. *Curcumin pre- and post-fibrosis induction treatment reduced oxidative stress in fibrotic rat liver. A. Lipid hydroperoxide (calculated as MDA level) in the liver of CCl_4-induced rats was increased significantly during the period of CCl_4-induced group vs. the pre-cur & CCl_4-induced experimental rats. Treatment with curcumin after fibrosis induction was shown to significantly reduce the MDA level (p < 0.05) in both groups at weeks 20 and 24 of the follow up period. B and C. GSH level and GPX activity were reduced significantly in CCl_4-treated rats (CCl_4-induced), while pretreatment with curcumin (pre-cur & CCl_4-induced) reduced the CCl_4-mediated oxidative stress. Treatment with curcumin for 4 and 8 weeks significantly elevated (p <0.05) the GSH level and GPX activities in both groups.*

Curcumin treatment reduces oxidative stress in the liver of CCl_4-induced rats

Curcumin treatment reduced lipid hydroperoxide (calculated as MDA level) production in the liver of CCl_4-induced rats. A significant increase in MDA level during CCl_4-injection *vs.* the pre-cur & CCl_4-induced experimental rats was observed. Treatment of fibrotic rats with curcumin was shown to significantly reduce the lipid peroxidation level (p< 0.05) in both groups at weeks 20 and 24 of the follow up period (Figure 3A).

GSH level and GPX activity were reduced significantly in CCl_4-treated rats, while pretreatment with curcumin (pre-cur & CCl_4-induced) reduced the CCl_4-induced oxidative stress (Figure 3B and 3C). Treatment with curcumin for 4 and 8 weeks after CCl_4 administration for 16 weeks significantly elevated (p< 0.05) the GSH level and GPX activity in both experimental groups.

Modulation of sera and liver inflammatory cytokines levels during the course of fibrosis induction and curcumin treatment

Hepatic fibrogenesis is commonly associated with inflammation. Levels of inflammatory cytokines; TNF-α, TGF-β, and IL-6 were determined in serum
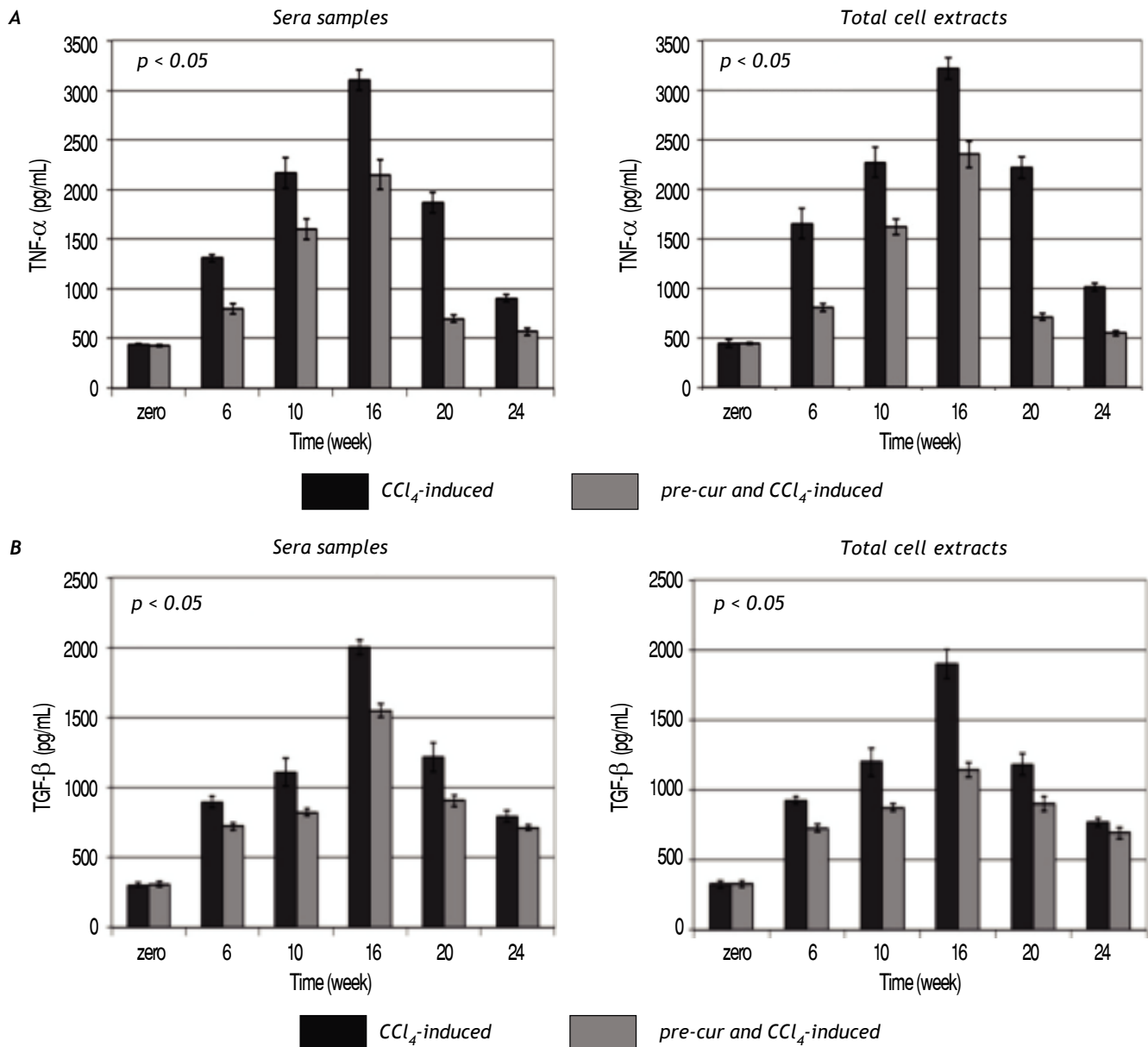
A **Sera samples**

B **Sera samples**

Figure 4. *Time course study of the change in TNF-α and TGF-β levels in sera and total cell extracts of CCl₄-induced and curcumin-treated groups. Data indicate significant elevation (p < 0.05) in both TNF-α (A) TGF-β (B) levels sera samples and cell extracts during the follow up weeks fibrotic rat liver versus control group. Pre-cur administration ameliorated the CCl₄-induced hepato-toxicity as indicated in group 3 compared to group 2. A significant reduction (p < 0.05) in both cytokines levels was observed at weeks 20 and 24.*

and liver homogenates. TNF-α, TGF-β, and IL-6 levels were elevated significantly (p< 0.05) during the 16 weeks of fibrosis induction compared to the control group (Figures 4 and 5). Pre-treatment with curcumin reduced the CCl$_4$-induced HSCs activation and inflammation as indicated by marked reduction in TNF-α, TGF-β, and IL-6 levels during the course of fibrosis induction compared to CCl$_4$-induced

rats. Curcumin treatment of both groups (group 2 and 3) induced-hepatocytes regeneration and inhibition of HSCs activation and inflammation as indicated by significant reduction in sera and total cell extracts TNF-α, TGF-β, and IL-6 levels (p< 0.05) at weeks 20 and 24. In addition, NF-κB level in nuclear extracts of CCl$_4$-induced and curcumin pre- and post-fibrosis treated rats showed a significant

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A
Sera samples

p < 0.05

Total cell extracts

p < 0.05

B
Total cell extracts

p < 0.05

■ *CCl₄-induced*

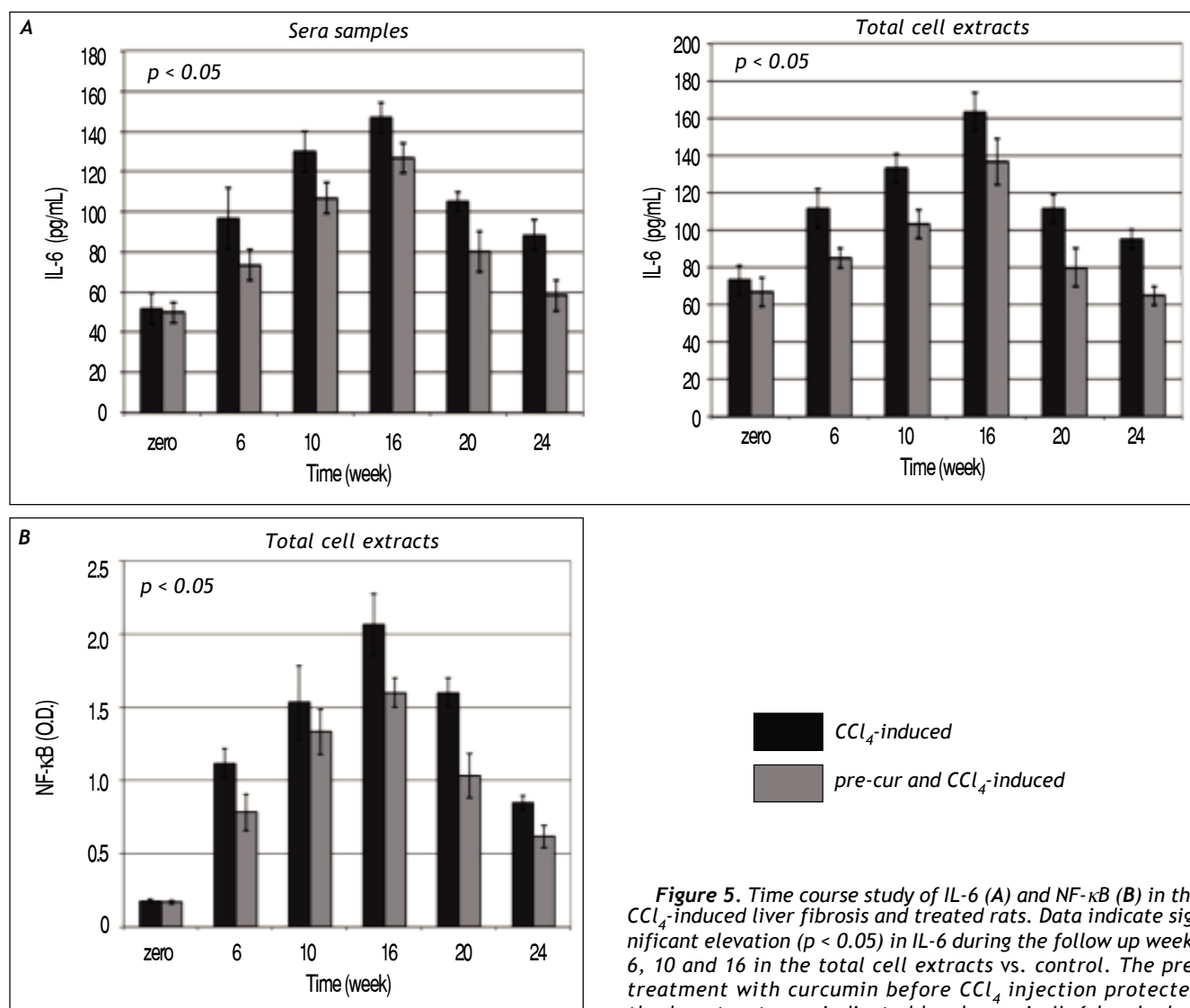▬ *pre-cur and CCl₄-induced*

Figure 5. *Time course study of IL-6 (A) and NF-κB (B) in the CCl₄-induced liver fibrosis and treated rats. Data indicate significant elevation (p < 0.05) in IL-6 during the follow up weeks 6, 10 and 16 in the total cell extracts vs. control. The pretreatment with curcumin before CCl₄ injection protected the hepatocytes as indicated by change in IL-6 level when compared to samples of group 2. Curcumin injection attenuated hepatic stellate cells activation and reduced IL-6 significantly (p < 0.05) in total cell extracts and sera samples at weeks 20 and 24. Also a significant elevation (p < 0.05) in NF-κB during the follow up period of fibrosis development in the rat liver versus control was observed. Pre-treatment and treatment by curcumin showed significant reduction (p < 0.05) in NF-κB at weeks 20 and 24.*

elevation ($p < 0.05$) during fibrosis development in the rat liver *vs.* control group. Treatment by curcumin reduced significantly ($p < 0.05$) NF-κB level at weeks 20 and 24 (Figure 5).

Curcumin modulates the expression of fibrotic vs. anti-fibrotic genes that are related to HSCs activation

We investigated the changes in the mRNA levels of pro-fibrotic genes (TGF-β, CTGF, TIMP-1, α-SMA, and STAP) and anti-fibrotic genes (MMP-2 and PPARγ) by reverse transcriptase-PCR.

Previous reports showed that curcumin inhibited *in vitro* HSCs activation by suppressing expression of genes relevant to cell growth and fibrogenesis.[19,30] Curcumin pre- and post-fibrosis induction treatment suppressed pro-fibrotic genes expression including TGF-β, CTGF, TIMP-1, α-SMA, and STAP and induced MMP-2. A marked elevation of TGF-β, CTGF, MMP-2, α-SMA, and STAP mRNA levels were observed as well (Figure 6). Pre-cur treatment ameliorated the CCl₄-induced HSCs activation and pro-fibrotic genes expression compared to rats in group 2 (Figure 6); 8 weeks of curcumin treatment for groups 2 and 3 reduced pro-fibrotic genes expres-
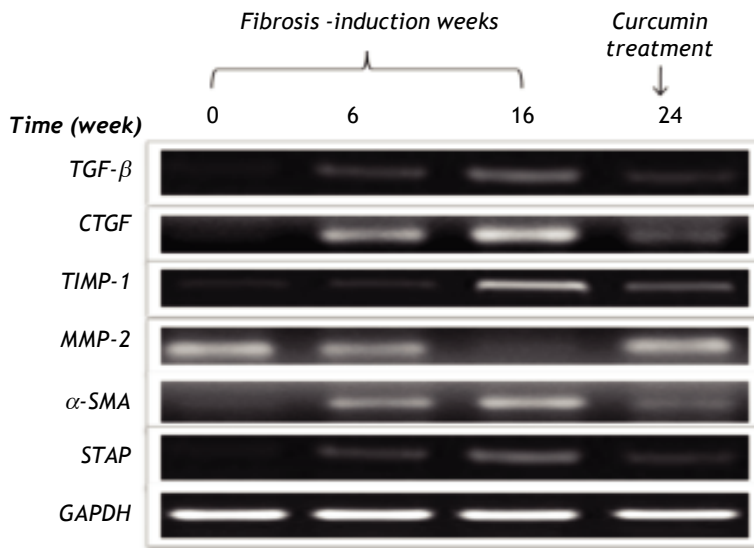
Figure 6. *Reverse transcriptase (RT)-PCR analyses of fibrosis* vs. *anti-fibrotic genes profile. The alterations in the mRNA levels were analyzed by RT-PCR. Curcumin modulates the expression of genes related to HSCs growth and fibrogenesis. mRNA levels of genes related to fibrogenic transformation, including, TGF-β, CTGF, TIMP-1, α-SMA, and STAP were significantly elevated during the CCL4-injection weeks. On the contrary, marked reduction in MMP-2 was observed. Curcumin treatment for 8 weeks reduced pro-fibrotic genes expression (TGF-β, CTGF, TIMp-1, α-SMA, and STAP) in both groups and induced increase in MMP-2 level. GAPDH was used as an invariant internal control.*

sion (Figure 6) and induced increase in MMP-2 level. Moreover PPARγ, which plays critical role during *in vivo* fibrogenesis and in HSCs during liver injury, was markedly reduced during CCl_4-induced fibrogenesis in group 2 (Figure 7). Pre-treatment with curcumin reduced the CCl_4-induced HSCs activation by maintain higher expression of PPARγ up to week 16 compared to group 2. PPARγ mRNA was re-elevated in both groups of fibrotic rats after curcumin treatment for 8 weeks (at week 24).

Curcumin anti-fibrotic effect may be correlated with alteration in APE1 mRNA and protein levels

In the present study we report for the first time that APE1 protein and mRNA levels are reduced significantly ($p < 0.05$) during the course of CCl_4-induced liver fibrosis (Figure 7 and 8). In addition to, in the pre-cur & CCl_4-induced group maintaining high level of APE1 expression was observed. Upon curcumin treatment of fibrotic livers for 8 weeks (week 24) APE1 mRNA and protein levels were re-elevated in both groups. The significant alteration in APE1 mRNA and protein levels of groups 2 and 3 (calculated as alterations of the controls; GAPDH and β-actin for mRNA and protein respectively) is represented in figure 7C and 7D. Also the percentage of fold change in APE1mRNA and protein levels of the control is shown (Figure 8). A significant re-

duction ($p < 0.01$) in APE1 both protein and mRNA levels from weeks 6 to 16 in CCl_4-induced (20 to 6% and 44 to 20% respectively) and pre-cur & CCl_4-induced groups (70 to 28% and 95 to 50%, respectively) was observed. Upon curcumin treatment a significant ($p < 0.01$) re-elevation in APE1 mRNA and protein levels was observed (30 and 95% in CCl_4-induced and 66 and 70% in pre-cur & CCl4-induced for mRNA and protein levels respectively).

Reduction in p53 mRNA and protein levels were observed during the course of fibrosis induction associated with elevated TGF-b mRNA and protein expression (Figure 7). Curcumin pre-treatment has protected rat liver during the first 6 weeks possibly by inducing apoptosis as confirmed by the presence of apoptotic bodies in histological examination and by detection of p53 expression at both mRNA and protein levels.

DISCUSSION

CCl_4 is one of the most widely used hepatic toxins for experimental induction of hepatic fibrosis and cirrhosis in laboratory animals. CCl_4-induced fibrosis or cirrhosis in experimental animals resembled human cirrhosis in some aspects of morphology and pathophysiology.[31] Thus, CCl_4-induced hepatic fibrosis due to stimulation of lipid peroxidation and the production of free radicals[32] has also been used to assess the efficacy of anti-fibrotic reagents and
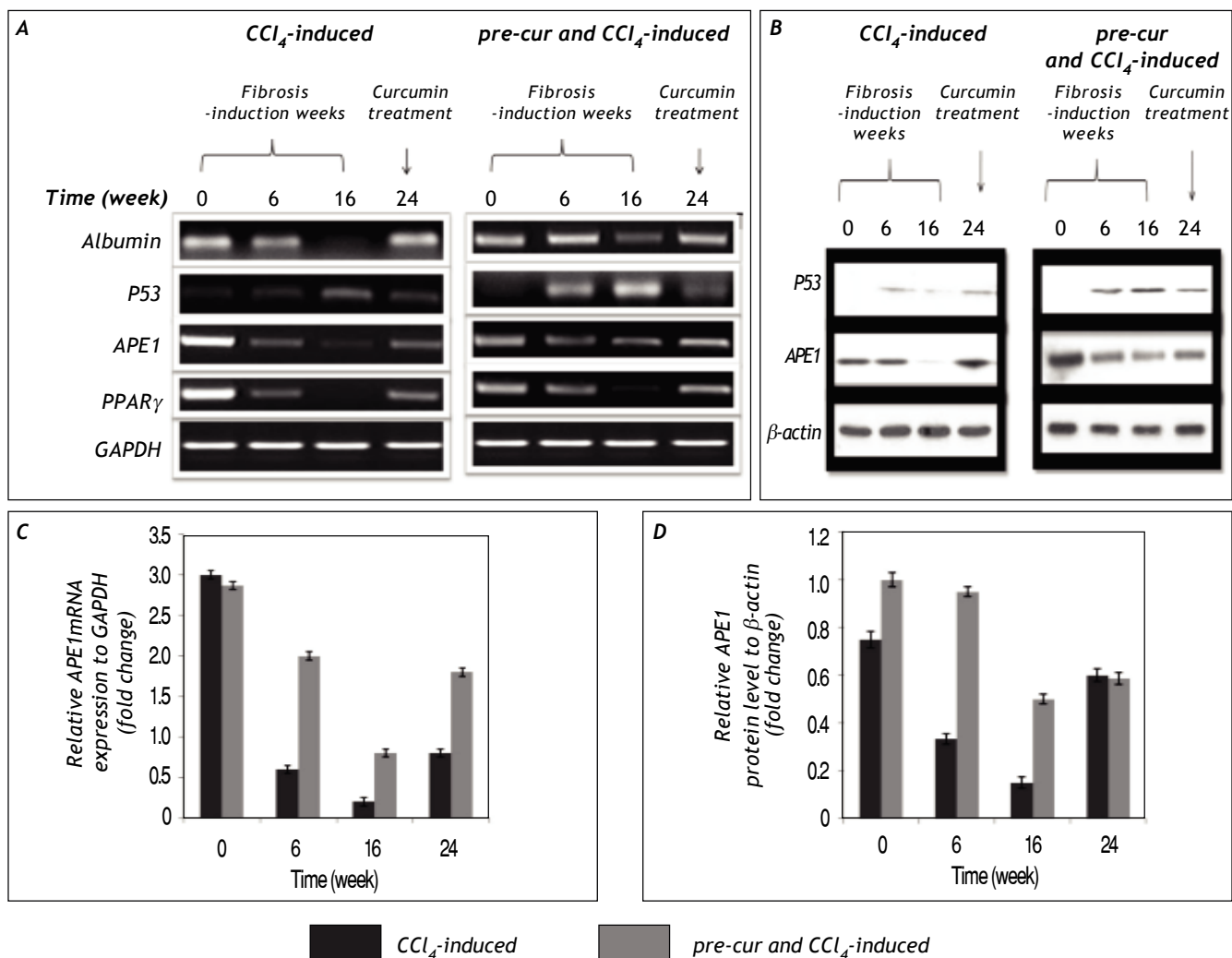
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Figure 7. *The change in p53 and APE1 mRNA and protein levels in CCl₄-induced fibrogenesis. **A** and **B**. Show the results of group 2 (CCl₄-induced) and group 3 (pre-cur treated & CCl₄-induced) of mRNA and protein levels respectively. Elevation in p53 mRNA level at weeks 6 to 16 of Cur- pre-treated & CCl₄-induced was detected. In addition, APE1 level is elevated at week 6 due to CCl₄-induced oxidative stress, its level is reduced significantly at week 16 and upon curcumin treatment for 8 weeks (week 24) a significantly induced APE1 mRNA and protein level (**A** and **B**) was observed. Moreover PPARγ, which plays crucial role during in vivo liver injury mRNA is reduced markedly during CCl₄-induced fibrogenesis in group 2 compared to less reduction in pre-cur treated & CCl₄-induced rats. Curcumin treatment attenuated the HSCs and possibly mediated an increase in APE1 and PPARγ mRNA levels at week 24. **C** and **D**. Represent the fold change in APE1 mRNA and protein levels in relative to GAPDH and β-actin levels respectively. Curcumin treatment may correlates with the significant (p< 0.05) expression of APE1 mRNA and proteins levels in cur- pre-treated & CCl₄-induced vs. CCl₄-fibrosis group. Representatives of three independent experiments are shown.*

to verify correlation between pathophysiologic feature of the liver and serum marker of fibrosis.[33]

In the present work, we have performed a study on a chronic model of liver fibrosis obtained by CCl₄ treatment combined with alcohol in drinking water during the last 4 weeks. We investigated therapeutic effects of curcumin on hepatic fibrosis and the variation of correlated cytokines. On this basis, oral administration of curcumin at 200 mg/kg dose significantly reduced elevated indices of liver, serum transaminases, and hyp contents in liver tissue. Histological examination showed curcumin evidently alleviated the progression of hepatic fibrosis. Therefore, intra-gavage administration of curcumin had apparently inhibitory effect on hepatic fibrosis induced by CCl₄ in rats.
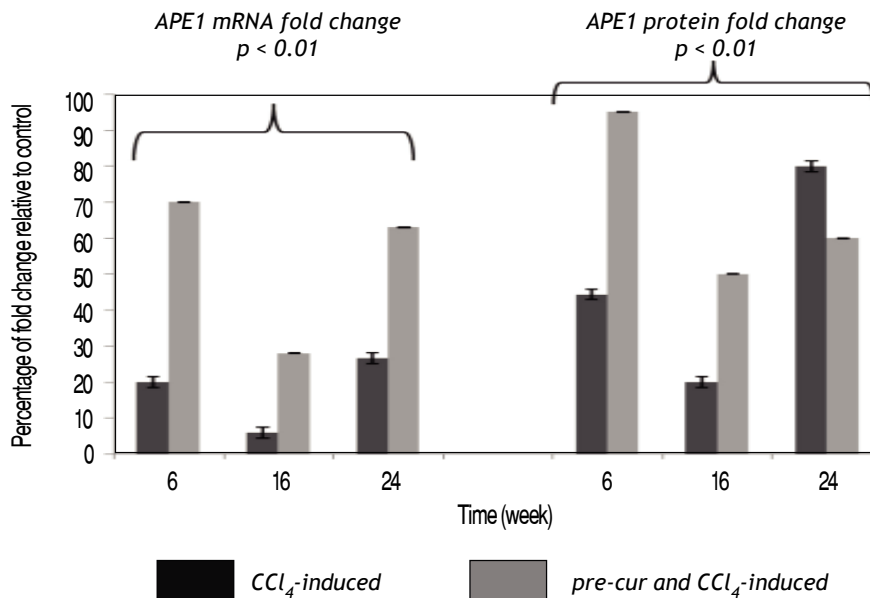
APE1 mRNA fold change
p < 0.01

APE1 protein fold change
p < 0.01

■ *CCl₄-induced*   ■ *pre-cur and CCl₄-induced*

Figure 8. *Fold change of APE1 mRNA and protein levels calculated as percentage of the control. Significant alterations (p < 0.01) in APE1 mRNA and protein levels calculated as fold change of the control values at zero week was observed in both groups. The significant reduction (p < 0.01) in APE1 mRNA and protein levels occurred during fibrosis induction by CCl₄ injection (at weeks 6 and 16; the % of fold change in mRNA level was 20 and 6% respectively and in protein level was 44 and 20% respectively) was significantly inhibited in pre-cur and CCl4-induced group (at weeks 6 to 16; the percentage of fold change in mRNA level was 70 to 28 respectively and in protein level was 95 to 50, respectively). While at week 24 where rats were treated with curcumin for 8 weeks the APE1 mRNA and protein levels markedly elevated in both groups as indicated.*

Herein we present the relationship between the oxidative stress induced HSCs activation, hepatic injury and fibrosis development and APE1/Ref-1. In addition, we examined p53 activity during CCl₄-induced liver fibrosis. We predicted APE1/Ref-1 would have an anti-fibrotic effect via effects on fundamental cellular antioxidant system. Our results indicated a significant elevation in the total antioxidant defense system activity with concomitant elevation in APE1 mRNA and protein levels at very early follow up time of fibrosis induction (weeks 2-4; data not shown). A significant reduction in APE1 mRNA and protein levels was observed during weeks 6, 10 and 16 of follow up period. The degree of reduction was milder in the group of rat pre-treated with curcumin before fibrosis induction (group 3). Due to chronic liver injury and chronic HSCs activation, p53 level was elevated only in group 3, rats pre-treated with curcumin followed by CCl₄ injection, consistently with appearance of apoptotic bodies in the microscopic examination.

Regulation of critical DNA repair enzymes such as APE1 in hepatic fibrosis presents a logical approach to therapeutic intervention against injury and fibrogenesis caused by carbon tetrachloride (CCl₄); however, the functional significance of the DNA repair pathways in CCl₄-induced hepatic fibrosis has not been directly tested. Although the mechanism was not completely clear, the present study suggests the unique finding that curcumin may act via induction of APE1 expression by unknown mechanism to afford protection to hepatic fibrosis.

APE1 is a critical cellular protein that performs multiple functions. In addition to its repair activity, APE1 acts as a transcriptional cofactor, as well as a suppressor of reactive oxygen species via a redox site.[34] Many of these functions could lead to hepatoprotection by curcumin.

Results from histopathological analyses of rats pre-treated with curcumin before CCl₄ injection (group 3) demonstrated area of apoptotic cells. In addition, results from determination of the level of hepatic injury-related biochemical markers indicated that the CCl₄-elevated partially the levels of hepatocytes biochemical markers. Curcumin treatment of experimental rats of groups 2 and 3 for 8 weeks induced hepatocytes regeneration and improved histological and biochemical results significantly.

Lipid peroxidation and necrosis are significantly suppressed in the liver of animals supplemented with antioxidants.[35] It was further demonstrated that de novo synthesis of GSH was a prerequisite for curcumin to inhibit HSC activation.[36] In this study, we hypothesized that curcumin might protect the liver against CCl₄-induced injury and fibrosis by attenua-

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ting oxidative stress. Curcumin attenuated oxidative stress as demonstrated by the reduction in the levels of lipid hydroperoxide in the CCl_4 rat model. These observations are supported by other studies. Our results in this study indicated that oral administration of curcumin not only increased the level of total hepatic GSH but also significantly improve activities of GST and catalase in the liver.

Pro-inflammatory TNF-α, IFN-γ, and IL-6 are major players in hepatic inflammation. TNF-α stimulates the development of hepatic fibrosis.[37] IL-6 produced by activated HSCs facilitates the production of ECM, including type I collagen, leading to hepatic fibrosis.[38] We reported in the present study that curcumin significantly reduced the levels of TNF-α, TGF-β and IL-6 in sera samples and the total cell extracts prepared form liver homogenates. As represented, sera and total cell extracts levels of TNF-α, TGF-β and IL-6 were significantly increased in the rats injected with CCl_4, and to a milder extent in the group of rats pre-treated with curcumin before CCl_4 injection *vs.* mock-treated group. Curcumin treatment for 8 weeks at the end of time course of fibrosis-induction, inhibited hepatic stellate cells activation and reduced the levels of TNF-α, TGF-β and IL-6 in sera samples and total cell extracts significantly ($p < 0.05$).

Further experiments revealed that curcumin enhanced the activity of antioxidant enzymes and modulated cytokines level by inducing expression of the genes. Activation of HSCs is triggered by various cytokines and chemokines, including the potent mitogens PDGF and EGF[39] released from Kupffer cells and activated HSCs and oxidative stress.[40,41] Results in this report further confirmed the *in vivo* effect of curcumin on the suppression of expression of these genes in the CCl_4 rat model demonstrated by semi-quantitative RT-PCR. In addition, HSCs activation coincides with a dramatic reduction in the abundance of PPARγ,[42,43] which might play an important role in hepatic fibrogenesis.

It has been previously demonstrated that curcumin dramatically induced gene expression of PPARγ in activated HSCs.[30] It was further reported that the interruption of the signaling pathways for TGF-β, PDGF, and EGF by curcumin was required for the curcumin induction of gene expression of PPARγ *in vitro.*[20] It was of interest to verify that the treatment with curcumin diminished the role of CCl_4 in the reduction of the number of PPARγ-positive HSC in the rat model. Our results demonstrated that curcumin protected the rat liver from CCl_4-caused injury and fibrogenesis *in vivo* by suppressing

hepatic inflammation, attenuating hepatic oxidative stress and inhibiting HSCs activation. The mRNA level of PPARγ was significantly reduced in the experimental rats of group 2 during the CCl_4-induced time course. Pre-treatment with curcumin prior to CCl_4 injection did not inhibit completely the expression of PPARγ compared to group 2. In addition treatment with curcumin after fibrosis induction in both groups, significantly elevated PPARγ mRNA level. These results confirm and extend our prior *in vitro* observations, and they provide novel insights into the mechanisms of curcumin in the protection of the liver.

In agreement with our work, Chen, et al. confirmed the previous work suggesting that curcumin activation of PPAR-γ results in the inhibition of NF-κB activation in HSCs.[8] In addition, they report that both NF-κB and the ERK MAP kinase pathway are required for the expression of CTGF, a key fibrogenic growth factor produced by HSCs. Furthermore, our results suggest and agree with this study that curcumin may suppress CTGF expression in HSCs by inhibiting the activation of NF-κB.

Collectively, our findings have important implications for understanding the pathophysiology of liver fibrosis and suggest that combined targeting of portal myofibroblasts activation could represent a central strategy to treat or delay the progression of chronic liver fibrosis. Curcumin can heal rat hepatic fibrosis. Effects of reducing the expression of correlated cytokines may be mechanisms of therapeutic effects of curcumin on hepatic fibrosis. This is in full agreement with recent studies.[44]

Our results show that curcumin may have multiple targets in liver, including inhibition of HSCs activation and inhibition of fibrotic signaling in portal myofibroblasts, thereby modulating several central cellular events in a rat model of liver disease. Further studies will be devoted to understanding the causal role of APE1 activation in curcumin hepatoprotective function.

ACKNOWLEDGEMENTS

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